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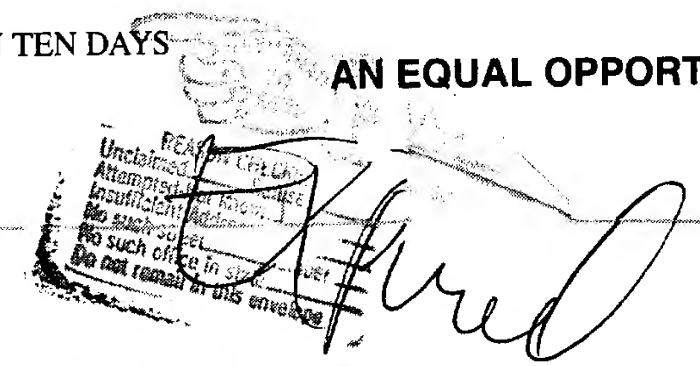

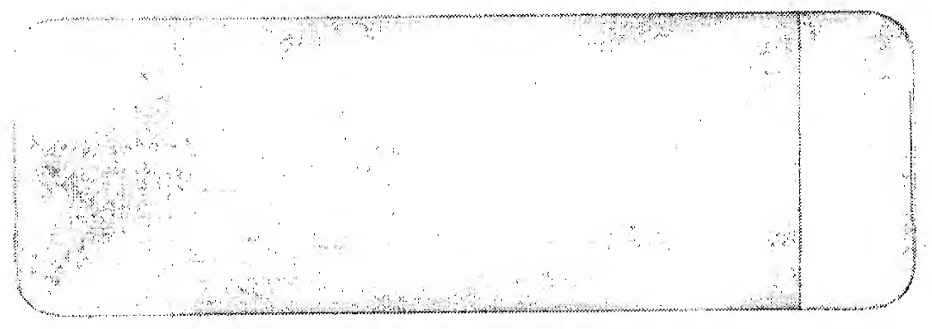

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/815,825	03/22/2001	Keith D. Allen	R-849	6413

26619 7590 04/07/2004

DELTAGEN, INC.
740 BAY ROAD
REDWOOK CITY, CA 94063

EXAMINER
SULLIVAN, DANIEL M

ART UNIT	PAPER NUMBER
1636	

DATE MAILED: 04/07/2004

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)	
	09/815,825	ALLEN ET AL.	
	Examiner	Art Unit	
	Daniel M Sullivan	1636	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *17 December 2003 and 16 January 2004*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-5,8-12,17-23,27-33,35,42,45 and 47* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☒ Claim(s) *4,5,8-12,17-23,27-33,35,42,45 and 47* is/are allowed.

6)☒ Claim(s) *1 and 3* is/are rejected.

7)☒ Claim(s) 2 is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: _____.

DETAILED ACTION

This Non-Final Office Action is a reply to the Papers filed 17 December 2003 and 16 January 2004 in response to the Non-Final Office Action mailed 26 August 2003. Claims 1-5, 8-12, 17-23, 27-33, 35, 42, 45 and 47 were considered in the 26 August Office Action. Claims 1-3, 5, 8, 10-12, 23, 32, 33, 35 and 42 were amended in the 16 January Paper. Claims 1-5, 8-12, 17-23, 27-33, 35, 42, 45 and 47 are pending and under consideration.

Response to Amendment

Claim Objections

Objection to claim 1 as containing informalities is withdrawn.

Claim Rejections - 35 USC § 112, First Paragraph

Rejection of claims 10, 11, 12, 23 32 and 35 under 35 U.S.C. 112, first paragraph, as lacking enablement for the full scope of the claimed subject matter is withdrawn in view of the amendment such that they are now limited to the subject matter enabled by the disclosure.

Rejection of claims 5, 8-12, 17-23, 27-33, 35, 42, 45 and 47 under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement is withdrawn in view of the limitation of the disruption of the claims to the absence of functional cGMP phosphodiesterase alpha subunit.

Rejection of claims 1-3 and 32 under 35 U.S.C. 112, second paragraph, as being indefinite is withdrawn.

New Grounds

Double Patenting

Applicant is advised that should claim 12 be found allowable, claims 27 and 32 will be objected to under 37 CFR 1.75 as being a substantial duplicate thereof. When two claims in an application are duplicates or else are so close in content that they both cover the same thing, despite a slight difference in wording, it is proper after allowing one claim to object to the other as being a substantial duplicate of the allowed claim. See MPEP § 706.03(k).

In the instant case, each of the claims is directed to a method limited to comprising the steps of administering an agent to the transgenic mouse of claim 8 and determining whether an eye abnormality of the transgenic mouse is ameliorated. There is no discernable difference in the scope of the claimed methods.

Claim Rejections - 35 USC § 112

The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 10 and 23 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

First, the claims omit essential steps, such omission amounting to a gap between the steps. See MPEP § 2172.01. The omitted steps are: Selecting a cell which has undergone homologous recombination following the step of introducing the targeting construct and prior to the step of introducing the cell into a blastocyst.

The claims are further indefinite in reciting in step (c), "said pseudopregnant mouse gives birth". Once the blastocyst has been implanted in the pseudopregnant mouse the mouse is no longer pseudopregnant. The mouse is actually pregnant. Amending step (c) to read "…wherein said mouse gives birth…" would be remedial.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Claims 1 and 3 are rejected under 35 U.S.C. 102(b) as being anticipated by Qin *et al.* (1992) *J. Biol. Chem.* 267:8458-8463.

Although it was previously indicated that the claims were free of the art, upon further consideration it is apparent that the targeting construct of claim 1 and the method of producing a targeting construct of claim 3 read on any nucleic acid comprising a nucleic acid encoding a cGMP phosphodiesterase and selectable marker because the selectable marker can be anywhere in the construct and the first and second sequences homologous to the cGMP phosphodiesterase alpha subunit gene are not limited to being non-contiguous. Thus, the nucleic acid construct

comprising a mouse cGMP phosphodiesterase and selectable marker gene, and method of making said nucleic acid construct, taught by Qin *et al.* anticipates the claims (see especially the second full paragraph on page 8459 and Figure 1 and the caption thereto.

Amending the claim to indicate that the first and second polynucleotide sequences homologous to the target gene are interrupted by the selectable marker gene and to indicate that the first and second sequences are inserted such that they flank the marker gene would overcome this rejection.

Allowable Subject Matter

Claim 2 is objected to as being dependent upon a rejected base claim, but would be allowable if rewritten in independent form including all of the limitations of the base claim and any intervening claims.

Claims 4, 5, 8-12, 17-23, 27-33, 35, 42, 45 and 47 are allowed.

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Daniel M Sullivan whose telephone number is 571-272-0779. The examiner can normally be reached on Monday through Thursday 6:30-5:00.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Remy Yucel, Ph.D. can be reached on 571-272-0781. The fax phone number for the organization where this application or proceeding is assigned is 703-872-9306.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

DMS

Anne-Marie Falk

ANNE-MARIE FALK, PH.D
PRIMARY EXAMINER

Notice of References Cited	Application/Control No. 09/815,825	Applicant(s)/Patent Under Reexamination ALLEN ET AL.	
	Examiner Daniel M Sullivan	Art Unit 1636	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Qin et al. In vitro isoprenylation and membrane association of mouse rod photoreceptor cGMP phosphodiesterase alpha and beta subunits expressed in bacteria. J Biol Chem. 1992 Apr 25;267(12):8458-63
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
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In Vitro Isoprenylation and Membrane Association of Mouse Rod Photoreceptor cGMP Phosphodiesterase α and β Subunits Expressed in Bacteria*

(Received for publication, November 6, 1991)

Ning Qin‡§, Steven J. Pittler¶‖, and Wolfgang Baehr‡¶**

From the Departments of ‡Biochemistry and ¶Ophthalmology, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030

We investigated the specificity of CAA*X* box-related isoprenylation of rod photoreceptor cGMP phosphodiesterase (PDE) subunits expressed in bacteria and the consequences of this modification on rod disk membrane association. Full-length cDNA sequences of the α and β subunits of mouse PDE, inserted into bacterial pET expression vectors, were overexpressed as fusion proteins containing 28 (bMP-α) and 26 (bMP-β) additional amino acid residues at their N termini. Both fusion proteins were overexpressed and stored in inclusion bodies. Purified bMP-α and bMP-β were recognized by bovine PDE-specific polyclonal antibodies, but did not associate with depleted rod disk membranes and were catalytically inactive. Using bovine brain or retina extracts as sources of protein prenyltransferases and tritiated farnesyl- or geranylgeranylpyrophosphate as donors, bMP-α (CAA*X* sequence CCIQ) was exclusively farnesylated, and bMP-β (CAA*X* sequence CCIL) was exclusively geranylgeranylated. After isoprenylation, bMP-α and bMP-β each associated with rod photoreceptor outer segment disk membranes under isotonic, but not under hypotonic, conditions. The results indicate that isoprenylated bMP-α and bMP-β each interact independently with membranes and that isoprenylation is the key modification that facilitates membrane association.

Rod photoreceptor cGMP phosphodiesterase (PDE),[1] a key component in mammalian phototransduction (1), is a heterotrimeric holoenzyme composed of two large subunits, α and β, of nearly identical size (calculated M_r 100,000) and two copies of a small inhibitory subunit γ (M_r 10,000) (2–4). Under physiological conditions, the holoenzyme is peripherally associated with ROS disk membranes, but may be solubilized reversibly by a membrane disrupting hypotonic shock (5, 6) or irreversibly by a limited trypsin treatment removing small C-terminal fragments from the large subunits (7–9). The two inhibitory γ subunits have no role in membrane association (9). Removal of γ results in a highly active αβ core enzyme, the two subunits of which cannot be separated without complete loss of activity.

Cloning of α and β subunit cDNAs revealed that each subunit is encoded by a distinct gene and that the predicted polypeptides are 70% identical (10–13). Each subunit has two presumed noncatalytic cGMP binding sites, an approximately 275-residue domain thought to represent at least part of the active site and C-terminal CAA*X* sequences where C is a Cys, A a mostly aliphatic residue, and *X* any residue (14). The CAA*X* motif (15, 16) appears to signal posttranslational isoprenylation of Cys via a thioether linkage, proteolytic C/AA*X* cleavage, and carboxymethylation of Cys-COOH in several eucaryotic proteins, including nuclear lamins (17), *ras*, and other small GTP-binding proteins (18), and their regulatory components (19, 20). By adding a hydrophobic tail, the modification enhances the hydrophobicity of the C terminus and thus may promote anchoring of the polypeptides to the membrane.

The isoprenoids transferred by specific protein prenyltransferases are the C_{15} moiety, farnesyl (21), and the C_{20} moiety, geranylgeranyl (22, 23). The specificity of isoprenylation by protein prenyltransferases was recently shown to be influenced by the last residue of the CAA*X* sequence (24, 25), where a Leu predetermines geranylgeranylation and Ser, Met, or Phe signal farnesylation in a variety of proteins thus far investigated (24, 26, 27). Accordingly, the γ subunit of transducin (CAA*X* sequence CVIS) has been shown to be farnesylated (28, 29). Although the mouse PDE β subunit (CAA*X* sequence CCIL) should be geranylgeranylated, the isoprenoid attached to the mouse PDE α subunit (CAA*X* sequence CCIQ) cannot be predicted on the basis of the identity of the last residue. Consistent with isoprenylation of the PDE subunits is the earlier finding that the mature α and β subunits are carboxymethylated (8, 9, 30). A lipid moiety has not been identified in either of the two large subunits.

We are interested in the structure and function of the two large subunits of PDE, α and β, representing the active core of the PDE holoenzyme $\alpha\beta\gamma_2$. We have initiated expression of α and β subunits in bacteria and yeast (31) to study membrane association, posttranslational modifications, and

* This investigation was supported in part by National Eye Institute Grant EY08123, the National Retinitis Pigmentosa Foundation, the Gund Foundation, and the Retina Research Foundation (Houston) (to W. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported as a predoctoral fellow by a center grant of the National Retinitis Pigmentosa Foundation to the Department of Ophthalmology.

‖ Supported by Individual National Research Service Award F32 EY06172 and a grant from the Knights Templar Eye Foundation, Inc.

** Recipient of a Jules and Doris Stein Research to Prevent Blindness Professorship (Research to Prevent Blindness, Inc., Baltimore). To whom correspondence should be addressed: Dept. of Ophthalmology, Baylor College of Medicine, 6501 Fannin St., Houston, TX 77030. Tel.: 713-798-5965.

[1] The abbreviations used are: PDE, phosphodiesterase; PCR, polymerase chain reaction; ROS, rod photoreceptor outer segments; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IPTG, isopropyl-1-thio-β-D-galactopyranoside; FPP, farnesylpyrophosphate; GGPP, geranylgeranylpyrophosphate; HPLC, high performance liquid chromatography.

interaction of the two large subunits. To determine the influence of isoprenylation of individual subunits upon membrane association, we describe in this paper the expression of full-length unprocessed fusion polypeptides bMP-α and bMP-β in bacteria. We show that bMP-α can be farnesylated and that bMP-β can be geranylgeranylated *in vitro* with absolute specificity. Although catalytically inactive, the isoprenylated fusion peptides readily associate with ROS membranes under isotonic, but not under hypotonic conditions, mimicking membrane association of native PDE.

EXPERIMENTAL PROCEDURES

Materials—[^{3}H]FPP (specific activity 1.4 Ci/mmol), [^{3}H]GGPP (8 Ci/mmol), and unlabeled FPP and GGPP were obtained from R. K. Keller (University of Florida). [^{3}H]cGMP (33.3 Ci/mol) was from Du Pont, and [^{35}S]methionine (1,000 Ci/mmole) was from Amersham Corp.; methyliodide, farnesol, and geraniol and alkaline phosphatase were from Sigma.

Full-length cDNA Clones Encoding Mouse PDE α and β Subunits—cDNA clones containing full-length coding sequences for the mouse PDE α subunit were constructed by ligating the N-terminal *Cla*I-*Xho*I cDNA fragment of MPA11 and the C-terminal *Xho*I-*Kpn*I fragment of MPA4 (13) at their common unique *Xho*I site. The ligation products were digested with *Kpn*I, and a 3.2-kilobase pair *Kpn*I-fragment containing the full coding sequence of the PDE α subunit was cloned into the *Kpn*I site of pBluescript. Plasmids containing the insert in both orientations (MPA-A and MPA-B) were isolated and characterized by restriction mapping and sequencing through the junctions used for ligation. Two cDNA clones (MPB-71 and MPB-81) containing full-length clones encoding the β subunit were isolated from a mouse retina λzap library using an N-terminal 250-base pair *Eco*RI-*Pst*I fragment of MPB10 (13) as a probe. The two clones differ in the extent of the 3′-untranslated area, and upstream of position −10 (position +1 is A of ATG), have differing 5′-untranslated regions, the basis of which is presently unknown.

Expression Constructs and Transformation—A 3.2-kilobase pair MPA fragment was isolated after digestion of MPA-B with *Cla*I, and a 2.8-kilobase pair MPB fragment was isolated after digestion of MPB-81 with *Eco*RI. The fragments were blunt-ended using the Klenow fragment of *Escherichia coli* DNA polymerase and dNTP. The MPA fragment was cloned into the filled-in *Bam*HI site of the bacterial expression vector pET-3c (32) to yield pETMPA, and the MPB fragment was cloned into the pET-3a vector to yield pETMPB. Correct orientations of both constructs were determined by restriction mapping, and the junctions were confirmed by DNA sequencing with PDE α and β subunit-specific antisense primers (data not shown). The constructs pETMPA and pETMPB were transformed into *E. coli* strain BL21(DE3)pLysS (F^- refA $r_{K12}m_{K12}r_b^-m_b^-$) (Novagen, Madison, WI). The transformants from both constructs were grown in 5 ml of LB medium containing 50 μg/ml ampicillin and 25 μg/ml chloramphenicol to CD_{600} = 1.0 and induced for 3 h by adding IPTG to a final concentration of 0.4 mM. Before and after induction, 100 μl of the culture were removed, spun down, and used directly for SDS-PAGE analysis.

Isolation of Native Bovine PDE and Expressed PDE Subunits—Bovine ROS PDE was isolated from 50 bovine retinas as a crude hypotonic supernatant as described previously (2). The supernatant was concentrated using ultrafiltration membranes (Diaflo, Amicon), dialyzed against 50% glycerol storage buffer (2), and stored at −20 °C. For solubilization of bacterially expressed proteins aggregated in inclusion bodies, cells from a 100-ml culture were harvested by centrifugation at 5,000 rpm (GSA rotor, Sorvall) and resuspended in 1/10 volume of lysis buffer (50 mM Tris/HCl pH 8.0, 1 mM EDTA, 0.5 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 20 mM β-mercaptoethanol), followed by freezing, thawing, and sonication (Branson). The lysate was centrifuged at 13,000 rpm (SA600 rotor, Sorvall) for 20 min and the precipitate washed with the same volume of lysis buffer. To the pellet, 2.0 ml of lysis buffer containing 8 M urea was added and incubated for 30 min at room temperature. Urea was removed by sequential dialysis against 2 M urea, 1 M urea in lysis buffer, and finally lysis buffer only. Five μl of the dialysate were used for SDS-PAGE. For storage, the bMP-α and bMP-β were dialyzed against 50% glycerol storage buffer and kept at −20 °C.

Protein Prenyltransferase—Bovine brain and retina extracts containing protein prenyltransferase activity were prepared from 10 g of brain tissue or five frozen retinas essentially as described by Reiss *et al.* (33). The 30–50% ammonium sulfate precipitate was dissolved in 5 ml of 20 mM Tris/HCl, pH 7.5, 1 mM dithiothreitol, 20 μM $ZnCl_2$, dialyzed against 1 liter of the same buffer, and finally against 100 ml of this buffer containing 50% glycerol. The recovered 1.5-ml extract was stored at −20 °C in several aliquots.

In Vitro Isoprenylation—The standard assay (33) mixture contained the following components in a volume of 10 μl: Tris/HCl, pH 7.5 (50 mM); $ZnCl_2$ (50 μM); KCl (20 mM); dithiothreitol (1 mM); a protease inhibiting mixture consisting of 1 μg/ml leupeptin, 1 μg/ml antipain, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, 0.2 mM phenylmethylsulfonyl fluoride; [^{3}H]FPP (1 μl, specific activity 1.4 μCi/pmol) or [^{3}H]GGPP (1 μl, specific activity 8 μCi/pmol); bMP-α or bMP-β (1.5 μg); brain extract (2 μl). After incubation at 37 °C for 1 h, 10 μl of 2 × SDS sample buffer were added, and the mixture was boiled and then separated by SDS-PAGE (2).

Metabolic Labeling of Bacterial Proteins—To label bacterial proteins with [^{35}S]methionine, 200 μl of a 2-ml overnight culture of pETMPA or pETMPB in BL21 cells were pelleted (2,000 rpm/5 min in Sorvall RT 6000B centrifuge) and washed with minimal M9 medium as described by Studier and Moffatt (34). The bacterial pellet was resuspended in 5 ml of M9 medium, grown to OD_{600} = 1.0, pelleted, and resuspended in M9 medium lacking $MgSO_4$. The culture was induced with 0.4 mM IPTG for 1.5 h, 50 μCi of [^{35}S]methionine were added, and labeling allowed to proceed for 0.5 h. Cells were spun down, and inclusion bodies were isolated and dissolved as described above.

Immunoprecipitation—Solubilized bMP-α and bMP-β were immunoprecipitated by a polyclonal anti-bovine PDE antibody and bound to protein-A-Sepharose CL-4B. Expressed polypeptides were eluted with 0.5 M NaCl, 0.1 M citric acid, pH. 4.2, and the eluates were neutralized and desalted with Centricon filters (Amicon).

SDS Gel Electrophoresis and Western Blots—SDS-PAGE was performed in 15% acrylamide, 0.08% bisacrylamide gels as described previously (2). For visualization of ^{3}H-labeled proteins, the gel was treated with EnHancer and exposed to Kodak XAR5 film at −80 °C. Proteins were transferred to nitrocellulose as described (35), using a Hoefer transfer unit for 2 h at 300 mA. The blot was processed using the Bio-Rad immunoblot (GAR-HRP) assay kit except that 5% nonfat dry milk was used as a nonspecific blocker (instead of 3% gelatin). The blot was incubated with polyclonal antibody (1:1,000 dilution) at 4 °C overnight.

HPLC Separation of Isoprenols—The chemical analysis of cleaved labeled isoprenols was performed essentially as described by Mumby *et al.* (19), except that trichloroacetic acid-precipitated isoprenylated samples were collected by centrifugation (instead of nitrocellulose filtration), and the methyliodide treatment was performed for only 8 h (instead of overnight). Labeled farnesol and geranylgeraniol standards were prepared by dephosphorylation of [^{3}H]FPP and [^{3}H]GGPP with alkaline phosphatase in 0.1 M glycine, pH 9 (37 °C for 30 min). Isocratic reverse phase HPLC was performed through an ODS-pak column (4.6 × 250 mm, Shodex) with 80% acetonitrile, 20% water for 30 min at a flow rate of 1 ml/min. The labeled isoprenols were detected in-line with a Flo-one\beta A-200 radioactivity detector (Radiomatic, Tampa, FL) and compared with the elution profile of authentic geraniol, farnesol, and geranylgeraniol monitored by UV absorbance.

Association of bMP-α and bMP-β with ROS Membranes—To test for membrane association (Fig. 5*A*), aliquots (20 μl, approximately 10,000 cpm) of non-isoprenylated metabolically labeled immunoprecipitated bMP-α and bMP-β were incubated with depleted ROS membranes (8 μg of Rho under isotonic conditions for 2 h at room temperature (final volume 100 μl). The membrane suspension was pelleted (10 min/15,000 rpm) and resuspended in 500 μl of isotonic buffer three times. Other aliquots of bMP-α and bMP-β were first isoprenylated *in vitro* as described above, incubated with ROS membranes under isotonic and hypotonic conditions, and resuspended/pelleted two times as described for non-isoprenylated material. Final pellets were resuspended in 10 μl of SDS sample buffer and subjected to SDS-PAGE and autoradiography.

RESULTS

Expression Constructs pETMPA and pETMPB—PETMPA was constructed by inserting 3.2 kb of cDNA encoding the mouse PDE α subunit (MPA, 859 amino acid residues) into the *Bam*HI site of the bacterial expression

vector pET-3c (32). In this vector, the ATG initiator of MPA (ATG^p, Fig. 1) is in-frame with the translation start codon of pET (ATG^b), resulting in a fusion protein bMP-α which contains 28 additional amino acids (Fig. 1). Similarly, a full-length cDNA encoding the β subunit (MPB, 856 residues) was inserted into the *Bam*HI site of pET-3a which differs from pET-3c by two nucleotides (32). The fusion protein bMP-β has 26 additional amino acids, the first 12 of which are identical with corresponding residues in bMP-α. Due to differences in the 5′-untranslated regions of MPA and MPB cDNAs, the residues immediately upstream of ATG^p differ. Both fusion proteins are expressed under the control of the T7-ϕ10 promoter (34) located 70 nucleotides upstream of ATG^b. The 3′-untranslated sequences of MPA and MPB are followed by a short stretch of bacterial untranslated sequence and a procaryotic transcriptional terminator.

Expression and Isolation of bMP-α and bMP-β—As observed for other bacterial expression systems (36), the majority of expressed fusion proteins bMP-α and bMP-β were stored in insoluble inclusion bodies. In Fig. 2 an SDS-PAGE analysis of crude bacterial lysates (*lanes 1–6*), and material solubilized from inclusion bodies (*lanes 7–9*), are shown. In the absence of inducer (*lanes* marked with −), only very small amounts of fusion proteins are expressed. In the presence of inducer (*lanes* marked with +), the level of expression of fusion protein, but not of other bacterial proteins, increases significantly. Urea/dithiothreitol treatment of isolated inclusion bodies, followed by dialysis and membrane concentration, resulted in a substantial purification of the fusion proteins (Fig. 2, *lanes 7* and *8*). The expressed fusion proteins (887 and 882 residues, respectively) exhibited the expected mobility on denaturing SDS gels, only slightly retarded compared with the subunits of native bovine PDE (*lane 10*). Both peptides were recognized by a polyclonal antibody which was raised against native bovine PDE, as shown by Western blotting (Fig. 2*B*). The antibody, which recognizes all three subunits of native PDE (*lane 10*), does not cross-react with any of the bacterial proteins. Some degradation of bMP-α and bMP-β in crude lysates (*lanes 4* and *6*) can be detected. The stability of both fusion proteins, however, was substantially improved after purification (*lanes 7–9*).

Isoprenylation of bMP-α and bMP-β—Native PDE α and β subunits were shown to be carboxymethylated (8, 9, 30), consistent with CAA*X* box-related posttranslational modifications. The chemical identity of the lipid presumed to be attached, however, has not been elucidated, and the questions as to whether α alone or β alone can bind to membranes after isoprenylation, and whether α mediates binding of β or *vice versa*, are unresolved. Our bacterial expression system allows purification of individual unprocessed fusion proteins bMP-α and bMP-β which are identical in sequence to native mouse PDE subunits except for short extensions of their N termini and lack of modification at the C terminus. We presumed that the fusion proteins would be substrates for protein prenyltransferases which have recently been identified in bovine brain and other tissues (37, 38) and that according to their different CAA*X* sequences (CCIQ for α, and CCIL for β), the isoprenoid modification would differ. As shown in Fig. 3, both bMP-α and bMP-β, can be isoprenylated *in vitro* with bovine brain extract as a source of protein prenyltransferase. When incubated with [^{3}H]FPP, only bMP-α (*lane 1*), but not bMP-β (*lane 2*), was labeled. In contrast, when incubated with [^{3}H]GGPP, only bMP-β (*lane 5*), but not bMP-α (*lane 4*), was labeled. Both isoprenylated fusion proteins could be immunoprecipitated with PDE antibody (*lanes 7* and *8*). As expected, native bovine PDE, which is most likely fully processed by isoprenylation and carboxymethylation, did not serve as a substrate (*lanes 3* and *6*). Experiments shown in *lanes 1–6* were successfully repeated with retina extracts as a source for prenyltransferase yielding a comparable labeling efficiency (not shown). The results show that brain and retina extracts contain specific protein prenyltransferases capable of transferring C_{15} and C_{20} isoprenoids to expressed fusion proteins and that the specificity of isoprenoid transfer is influenced by the last residue of the CAA*X* sequence.

HPLC Analysis of Isoprenoids Attached to bMP-α and bMP-β—Since we used crude bovine brain extracts to transfer FPP and GGPP to purified bMP-α and bMP-β, we verified the

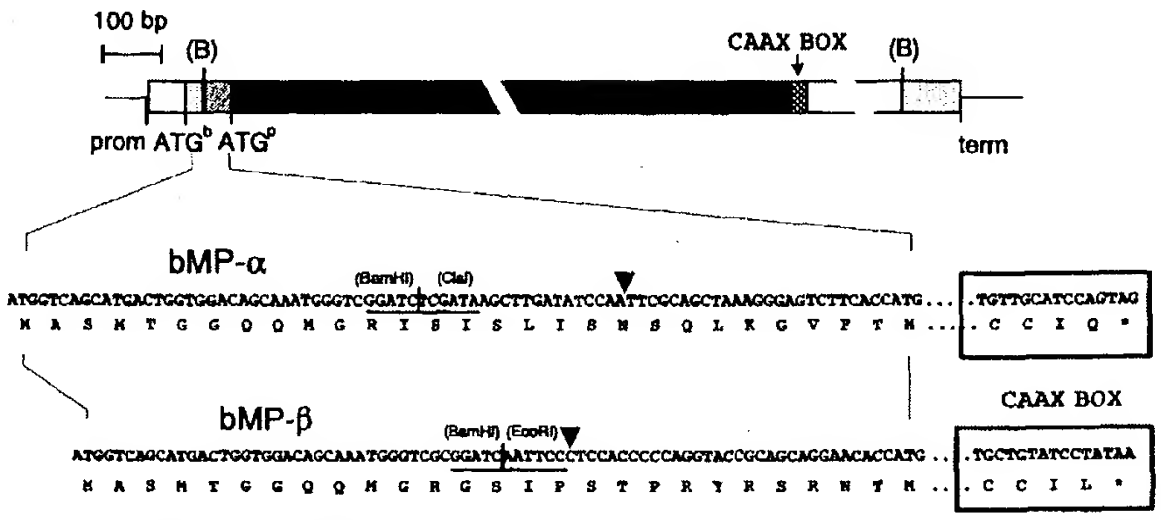

FIG. 1. **The pETMPA and pETMPB expression constructs.** *Top*, schematic representation of the construct. PDE sequences were inserted by blunt-end ligation into the *Bam*HI site (*B*) of the pET vector. *Black box*, PDE coding sequence. *Cross-hatched box*, CAA*X* box. *White boxes*, untranslated regions. *ATG*^{b}, bacterial translation start codon; *ATG*^{p}, translation start codon of PDE subunit. *Stippled box* between ATG^b and *B*, N terminus derived from pET. *Hatched box* between *B* and ATG^p, sequence deriving from multiple cloning site of bluescript and 5′-flanking region of MPA. *Prom*, T7-ϕ10 promoter; *term*, transcription terminator. *Bottom*, the sequences of the fusion proteins of bMP-α and bMP-β. (*BamHI*)/(*ClaI*) indicates the junction between the pET vector and MPA-B and *(BamHI)/(EcoRI)* the junction between pET and MPB-81. Due to blunt-end ligation, the restriction sites were not recovered. Triangles (▼) depict the border between Bluescript multiple cloning site and the 5′-flanking sequences. Of the native PDE subunit sequences, only ATG and the 12 C-terminal nucleotides encoding the respective CAA*X* boxes are shown.

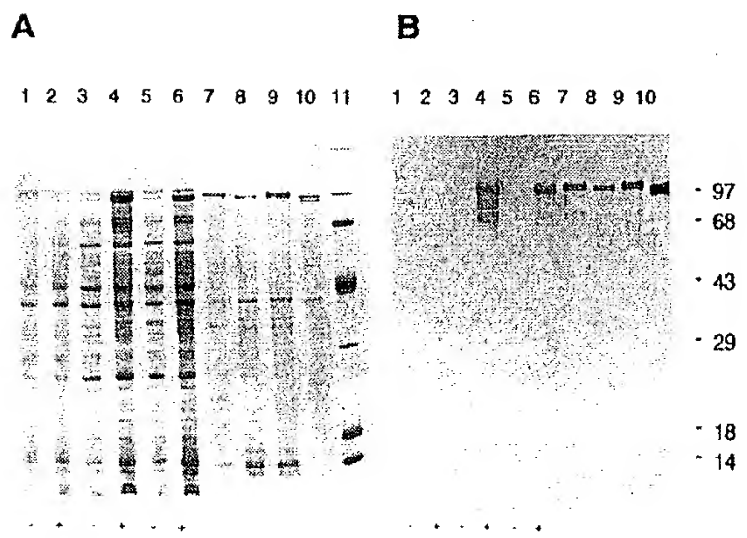

FIG. 2. **SDS-PAGE and Western blot of expressed fusion proteins (bMP-α and bMP-β).** The − and + signs below the gel indicate cultures uninduced and induced by IPTG, respectively. *Lanes 1* and *2*, polypeptides of lysate containing pET vector only (no insert). *Lanes 3* and *4*, polypeptides of lysates containing pETMPA. *Lanes 5* and *6*, polypeptides of lysates containing pETMPB. *Lane 7*, bMP-α and *lane 8*, bMP-β, both solubilized from inclusion bodies. *Lane 9*, a 1:1 mixture of bMP-α and bMP-β. *Lane 10*, 2 μg of concentrated hypotonic supernatant of bovine ROS containing native PDE. *Lane 11*, molecular weight markers (Bethesda Research Laboratories). *B*, Western blot of gel shown in *A*, excluding *lane 11*. The polyclonal antibody used recognizes all three native PDE subunits (*lane 10*).

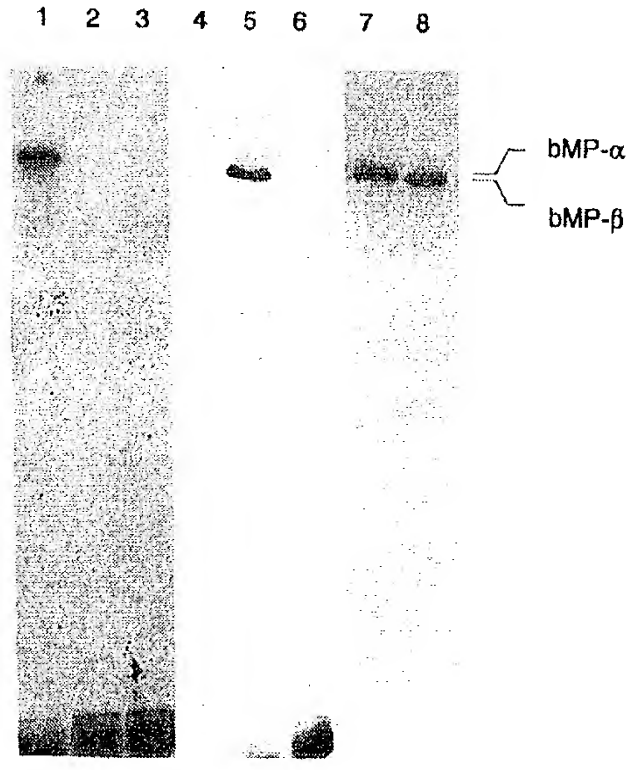

FIG. 3. ***In vitro* isoprenylation of bMP-α and bMP-β with [^{3}H]FPP and [^{3}H]GGPP.** Purified bMP-α, bMP-β, and native PDE as a control were isoprenylated in the presence of [^{3}H]FPP (*lanes 1–3*) or [^{3}H]GGPP (*lanes 4–6*) and bovine brain protein prenyltransferase, separated by SDS-PAGE, and fluorographed. *Lane 1*, bMP-α/FPP; *lane 2*, bMP-β/FPP; *lane 3*, native bovine ROS PDE/FPP; *lane 4*, bMP-α/GGPP; *lane 5*, bMP-β/GGPP; *lane 6*, native PDE/GGPP. Isoprenylated samples shown in *lanes 1* and *5* were immunoprecipitated with a PDE monospecific polyclonal antibody, and the precipitates were solubilized and rerun (*lanes 7* and *8*).

identity of the lipids attached by HPLC following methyliodide treatment, a procedure which specifically cleaves thioethers producing isoprenols. Shown in Fig. 4*A* is the HPLC profile of the isoprenoid released from bMP-α^f. The major product exhibits a retention time of 10 min, identical to that observed for authentic [^{3}H]farnesol, as prepared by phosphatase cleavage of [^{3}H]FPP. The lipid moiety released from bMP-β^g exhibits a retention time identical to [^{3}H]geranylgeraniol (Fig. 4*B*). Minor peaks and shoulders accompanying the authentic C_{15} and C_{20} moieties, most likely isomers, were also observed in samples obtained by phosphatase treatment of [^{3}H]FPP and [^{3}H]GGPP (not shown). The results show that isoprenoids are transferred by brain protein prenyltransferases without further modification and covalently linked as a thioether to the expressed fusion proteins.

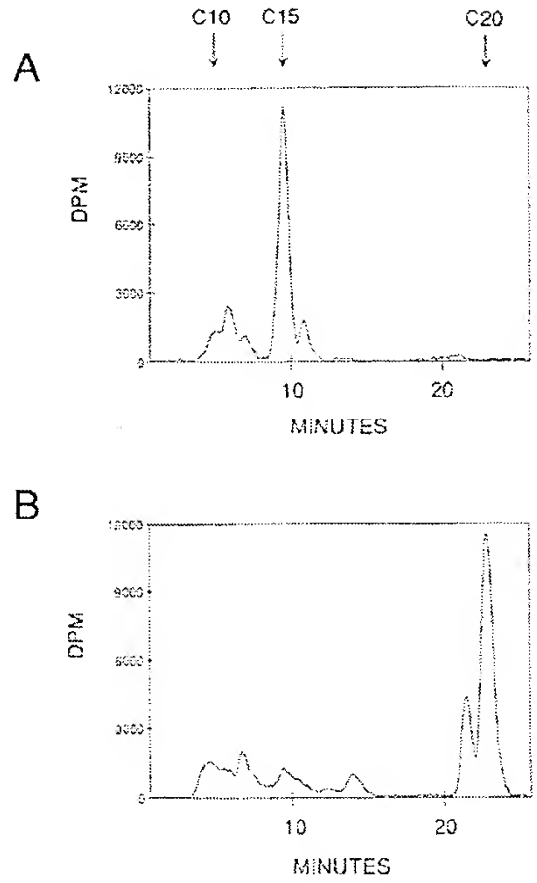

FIG. 4. **HPLC profiles of labeled isoprenols after cleavage of isoprenylated bMP-α and bMP-β with trypsin/methyl iodide.** The elution positions of authentic labeled and unlabeled standards, geraniol, farnesol, and geranylgeraniol are indicated by *arrows*. *A*, isoprenoid (farnesol) obtained from cleaved isoprenylated bMP-α; *B*, isoprenoid (geranylgeraniol) obtained from isoprenylated cleaved bMP-β.

Membrane Binding of Isoprenylated Fusion Proteins—To examine the influence of isoprenoid addition on membrane binding, we first metabolically labeled bMP-α and bMP-β with [^{35}S]methionine and then isoprenylated the immunoprecipitated ^{35}S-labeled bMP-α and ^{35}S-labeled bMP-β with unlabeled FPP and GGPP, respectively (Fig. 5*A*). The non-isoprenylated and isoprenylated immunoprecipitated fusion proteins were then incubated with depleted ROS membranes, respectively, under conditions which promote membrane association (isotonic buffer) and under conditions which disrupt membrane association (hypotonic buffer) (2). The results demonstrate (Fig. 5*B*) that the non-isoprenylated (unprocessed) fusion proteins do not associate with membranes (*lane 4* for bMP-α and *lane 8* for bMP-β). Isoprenylated fusion proteins, in contrast, bind to membranes (*lanes 5* and *9*, respectively) under osotonic conditions, but do not bind under hypotonic conditions (*lanes 6* and *10*, respectively).

DISCUSSION

Membrane association of PDE (39) is reversible and thought to be mediated by immersion of a hydrophobic tail into the phospholipid bilayer of ROS membranes. The open questions concerning membrane association of PDE at the beginning of our studies were which of the two large subunits (or both?) would provide the hydrophobic tail to be inserted, and what is its chemical nature? Our approach to answer these questions was to express the two large subunits in

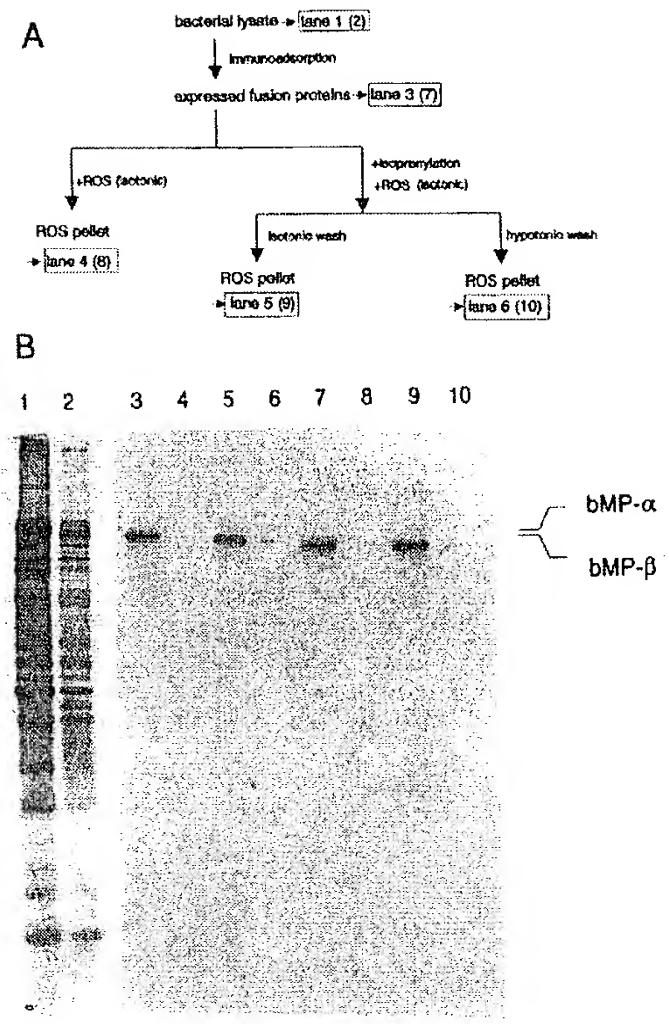

FIG. 5. **Membrane binding of isoprenylated [^{35}S]methionine-labeled bMP-α and bMP-β.** *A*, schematic of procedure used to show membrane binding of isoprenylated fusion proteins. *B*, SDS-PAGE analysis and autoradiography. *Lanes 1* and 2, bacterial lysates of IPTG-induced pETMPA and pETMPB cultures, respectively, which have been labeled metabolically with [^{35}S]methionine. *Lanes 3* and 7, immunoprecipitation of lysates shown in *lanes 1* (bMP-α) and 2 (bMP-β). *Lanes 4* and 8, membrane pellets of depleted ROS incubated in isotonic buffer with non-isoprenylated bMP-α and bMP-β (no binding). *Lanes 5* and *9*, membrane pellets of depleted ROS incubated with farnesylated bMP-α (bMP-α^f) and geranylgeranylated bMP-β (bMP-β^g) in isotonic buffer (binding). *Lanes 6* and *10*, pellets of ROS membranes incubated with bMP-α^f and bMP-β^g in hypotonic buffer (no binding).

bacteria where protein prenylation does not occur and to study membrane association of expressed proteins before and after isoprenylation *in vitro*. We decided to use the pET expression system to produce fusion proteins with 26–28 N-terminal amino acid extensions (Figs. 1 and 2). As subunits to be expressed we chose mouse PDE α and β subunits, which we had cloned previously as full-length copies (13, 40). The mouse PDE α and β subunit sequences are more than 90% identical with corresponding sequences in bovine, the system in which most of the PDE biochemistry has been done, and membrane binding properties of mouse and bovine PDE are presumed to be identical. As expected, the expressed fusion proteins did not bind in isotonic buffer to ROS membranes (Fig. 5) which were depleted of other peripheral membrane proteins by exhaustive treatment with hypotonic buffer. A crude brain extract, an ammonium sulfate cut containing many other enzymes including farnesyl and geranylgeranyl transferases, farnesylates bMP-α, and geranylgeranylates bMP-β (Fig. 3). The individual isoprenylated proteins readily associate with ROS membranes under isotonic conditions, but do not bind in hypotonic conditions, mimicking the membrane binding behavior of native PDE. We have not tested the isoprenylated fusion proteins for C/AA*X* cleavage. Since carboxymethyltransferase and C/AA*X* carboxypeptidase are particulate (41, 42) and may be present in depleted ROS membranes, partial processing cannot be excluded. In the absence of *S*-adenosylmethionine as a methyl donor, however, we assume that the isoprenylated fusion proteins are not carboxymethylated.

The CAA*X* box sequences of the expressed fusion proteins (and of the native PDE α and β subunits) differ only in their C-terminal residues (CCIQ *versus* CCIL, Fig. 1). Our finding of specific C_{15} isoprenoid transfer for bMP-α and C_{20} transfer for bMP-β, coupled with previous findings that the CAA*X* sequence itself is sufficient to signal prenylation (27), confirms the presumption that the C-terminal residue *X* of CAA*X* is the sole determinant of prenyl transferase specificity (43, 44). Although most naturally occurring CAA*X* substrates for farnesylation carry an S, M, or F as the last residue (*X*) (24, 27), a synthetic CCVQ peptide inhibited protein farnesylation to a similar extent as CVLM or CVLS (27). Since geranylgeranylation of bMPα is undetectable in our assay (Fig. 3, *lane 2*), we conclude that *X* = Q in CCI*X* specifically signals farnesylation. Our results also show that like brain, the retina, where rod PDE is exclusively located, contains farnesyl and geranylgeranyl transferase activities, consistent with the nearly ubiquitous occurrence of protein prenyltransferases in mammalian cells.

Although both subunits contain a C-terminal domain that is thought to contain at least part of the active site, native α and β cannot be separated under nondenaturing conditions. Preliminary attempts to recover a fusion protein bMP-α or bMP-β that is capable of hydrolyzing cGMP, or to reconstitute an active enzyme from both expressed subunits in a stoichiometric ratio, were unsuccessful. Taken with previous results, in which a truncated β subunit leads to an inactive PDE holoenzyme in the *rd* mouse (45), individual subunits most likely will not exhibit catalytic activity. Our results indicate that bacterially expressed large subunits of PDE, and hence most likely the native subunits, are posttranslationally isoprenylated by farnesyl and geranylgeranyl groups, respectively, and that both subunits may have independently the ability to associate with membranes under appropriate conditions. In the simplest model, farnesyl and geranylgeranyl tails would provide the molecular anchors. Recent experiments, however, in which soluble native PDE was subjected to limited trypsinolysis (46) indicated that C-terminal cleavage of the β subunit initiated the loss of membrane binding favoring a model in which α is largely bound to the membrane through association with β. It appears possible that the farnesyl tail interacts more weakly with membranes than the geranylgeranyl tail (22). Rhodopsin kinase, predicted to be farnesylated (CAA*X* sequence CVLS), interacts only weakly with membranes (47). Cone PDE, consisting of only one large subunit α', is predicted to be geranylgeranylated (CAA*X* sequence CLML) and known to be membrane associated under isotonic, but not hypotonic conditions (48, 49). Since the bacterially expressed polypeptides do not assume a native conformation, the possibility cannot be ruled out that the farnesyl group on the native α-subunit is oriented differently or even away from the membrane, and therefore, could be involved in function other than membrane binding. Isoprenylation of the transducin γ subunit, for example, has been shown to be necessary for GDP/GTP exchange on the transducin α subunit (28), consistent with a role beyond membrane association.

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